

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
11 January 2001 (11.01.2001)

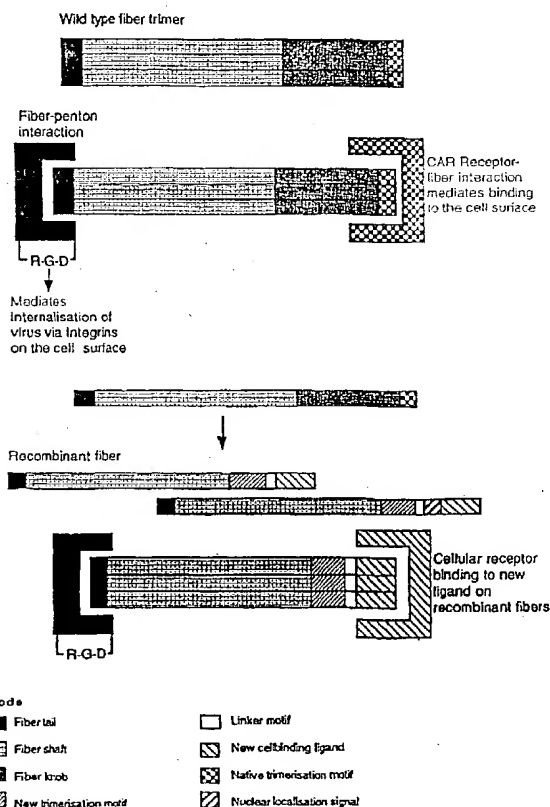
PCT

(10) International Publication Number
WO 01/02431 A1

- (51) International Patent Classification⁷: C07K 14/075, C12N 7/00, 15/00
- (21) International Application Number: PCT/SE00/01390
- (22) International Filing Date: 30 June 2000 (30.06.2000)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:
9902601.5 6 July 1999 (06.07.1999) SE
60/143,632 14 July 1999 (14.07.1999) US
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- (81) Designated States (national): AE, AG, AL, AM, AT, AT (utility model), AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, CZ (utility model), DE, DE (utility model), DK, DK (utility model), DM, DZ, EE, EE (utility model), ES, FI, FI (utility model), GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KR (utility model), KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SK (utility model), SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

[Continued on next page]

(54) Title: RECOMBINANT ADENOVIRUS



(57) Abstract: Recombinant adenovirus with changed tropism. In the adenovirus the native pentone fibre, comprising a fibre tail, a fibre shaft and a fibre knob including a trimerisation motif, has been changed in that the native knob containing the cell binding structure and the native trimerisation motif has been removed and a new cellbinding ligand and an external trimerisation motif have been introduced into the virus fiber. Further, the invention relates to the recombinant adenovirus for the treatment of human diseases, either in vivo or by in vitro methods and also to a method for rescuing of recombinant adenovirus fibers into the adenovirus genome.

WO 01/02431 A1



(84) **Designated States (regional):** ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:

— With international search report.

— Before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments.

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

RECOMBINANT ADENOVIRUSField of the invention

The present invention relates to new recombinant
5 adenovirus with changed tropism. More particularly the
recombinant adenovirus has been constructed by removing
the native knob structure and replacing it with a new
cell binding ligand and an external trimerisation motif.
The invention also relates to the new adenovirus for
10 treatment of human diseases. Also included is a method
for rescuing of recombinant adenovirus fibers into the
adenovirus genome.

Background of the invention.

15 Clinical gene therapy was introduced in 1989. The aim at
that time was to correct a gene defect in the immune
system through the in vitro introduction of a healthy
gene into the defect cells of the patient and transfusion
20 of the treated cells back to the patient. Since that
time, the possible indications for gene therapy have
increased dramatically. Today, ten years after its
introduction, the use of gene therapy to treat e.g.
diseases of the blood vessels, cancer, inflammatory
25 diseases and infectious diseases such as HIV can be
envisaged.

At present, however, gene therapy is not a useful method
in human medicine. One main reason is that gene therapy
30 demands the packaging of the genes to be delivered into
gene-carriers, or vectors, which can be injected into

patients and which will target the genes only to the intended cells. Such vectors have so far not been available.

- 5 Adenoviruses (Ad) are DNA viruses without an envelope, shaped as regular icosahedrons with a diameter of 60-85 nm. Cell-binding takes place through fiber proteins, anchored to the virion at the corners of the icosahedron. The fiber protein is not necessary for assembly and
10 release of intact virions. Assembly of virions take place in the nucleus of infected cells.

The fiber protein, which is a homotrimer of a fiber polypeptide, contains three functionally different parts:
15 an N-terminal tail anchoring the fiber non-covalently to the penton base in the virion and which furthermore contains the nuclear-localization signal; an approximate 15 amino acid fiber shaft motif which is repeated six times in Ad3 and 22 times in Ad2 and Ad5 (Chrobozek J,
20 Ruigrok RWH and Cusack S: Adenovirus Fiber, *Current Topics in Microbiology and Immunology*, 1995, p 163-200); and a C-terminal globular domain, the knob, which contains the ligand which binds to the cellular Ad-receptor (See review in in the previous ref.). Each shaft
25 repeat has two three-amino acid regions which form β -sheets and two amino acid regions which constitute the turns of the native extended fiber shaft. The crystal structure of the trimerised, cell-binding domain has been determined and shows a unique topology different from
30 other anti-parallel β -sandwiches (Di Xia, Henry LJ, Gerard RD and Deisenhofer J: Crystal structure of the receptor-binding domain of adenovirus type 5 fiber protein at 1.7 Å resolution, *Structure* 2: 1259-1270,

1994.). Binding of the fiber to the penton base of the virion can take place also in a cell-free system, i.e. the fiber can bind to fiberless virions (Boudin M-L and Boulanger P: Assembly of Adenovirus Penton Base and Fiber, *Virology*, 116: 589-604, 1982).

It seems possible that the fiber can tolerate structural modifications as long as the ability to bind to the penton base and to be transported to the nucleus is retained. Some attempts at modifying the Ad fiber in order to change the binding properties of the virus have been made. A short peptide ligand has been added C-terminally of the knob (Michael SI, Hoy JS, Curie DT and Engles JT: Addition of a short peptide ligand to the adenovirus fiber protein. *Gene Therapy* 2: 660-8, 1995.) and an octapeptide has been introduced into one of the knob "loops". By introducing the FLAG tetra-amino acid motif into the Ad penton, it has been shown possible to target Ad to cells normally not infected by Ad. This was done by targeting with bi-specific antibodies where one specificity was directed against the FLAG peptide and the other against the new target cell (Wickham TJ, Segal DM, Roelvink PW, Carrion ME, Lizonova A, Lee GM and Kovesdi I: Targeted Adenovirus Gene Transfer to Endothelial and Smooth Muscle Cells by Using Bispecific Antibodies. *J. Virol.*, 70: 6831-6838, 1996.). It would therefore seem possible to target Ad to a broad range of human cells which would be very useful for the purpose of human gene therapy. For these reasons and for the reason that Ad have been used extensively for gene therapeutic applications (Trapnell BC and Gorziglia: Gene therapy using adenoviral vectors, *Current Opinion in Biotechnology*, 5: 617-625, 1994.) a method has now been

developed to create recombinant re-targeted Ad-virus which can be useful for human gene therapy.

Accordingly it is an object of the present invention to
5 provide a recombinant adenovirus with changed tropism.

Another object of the invention is the recombinant adenovirus for treatment of human diseases.

10 A further object of the invention is a method for rescuing of recombinant adenovirus fibers into the adenovirus genome.

Summary of the invention

15

The objects of the invention are obtained by the recombinant adenovirus and the method for rescuing the virus fibers as claimed in the claims.

20 According to the invention there is provided a recombinant adenovirus with changed tropism. The adenovirus is characterized in that the native pentone fibre, which comprises a fibre tail, a fibre shaft and a fibre knob including a trimerisation motif, has been
25 changed in that the native knob containing the cell binding structure and the native trimerisation motif has been removed and a new cellbinding ligand and an external trimerisation motif have been introduced into the virus fiber.

30

The structural modification has been performed by DNA technology at the gene level or by chemical or immunological means at the virus level.

35 According to another aspect of the invention adenovirus, as identified above, is used for the treatment of human diseases, either in vivo or by in vitro methods.

A further aspect of the invention is a method for rescuing of recombinant adenovirus fibers into the adenovirus genome comprising the following steps:

- 5 a) subcloning of a 9kb fragment (from SpeI to end of genome),
- b) further subcloning of a 3kb fragment between SacI and KpnI,
- 10 c) deletion of the fibergene between NdeI and MunI and replacing the missing sequence with SEQ ID NO: 13 in the Sequence listing containing an XhoI site;
- d) ligation of recombinant fiber between NdeI and XhoI of construct under c) above;
- 15 e) re-introduction of construct under d) above into the 9 kb fragment cut with NheI using homologous recombination in E. coli;
- f) isolation of the recombinant 9 kb fragment under e) and re-creation of the adenovirus genome by joining 9 kb
- 20 fragment to the 27 kb fragment from the beginning of the genome to the SpeI site by Cosmid cloning.

Detailed description of the invention

25 Figure legends

Fig. 1: Summary of modifications to native fiber carried out in the invention.

30 Fig. 2: Recombinant adenovirus fibers.

Fig. 3: Method for rescuing of recombinant fiber genes into the Ad genome.

35 Fig. 4a: Recombinant fibers rescued into Ad genomes which are capable of giving CPE/plaques on transfected cells and in secondary cultures.

Fig. 4b: Recombinant fibers rescued into Ad genomes which are capable of giving CPE/plaques on transfected cells and in secondary cultures.

5

In the present invention re-targeting of Ad is achieved through the introduction of a new cell-binding ligand into the fiber (Fig. 1). Any cell binding peptide can be used, e.g. a monoclonal antibody or a fragment thereof whether as a single chain fragment or Fab, a T cell receptor or a fragment thereof, an integrin binding peptide such as RGD or a growth factor such as Epidermal Growth Factor.

15 Ligands which so far have been applied include Epidermal Growth Factor (EGF), the amino acid motif RGD, a single chain fragment of a cloned T-cell receptor (scTCR) reactive with MAGE-1 peptides associated with HLA-A1 (vd Bruggen P, Traversaari C, Chomez P, Lurquin D, De Plaen E, vd Eynde B, Knuth A and Boon T: A Gene encoding an Antigen Recognized by Cytolytic T Lymphocytes on a Human Melanoma, *Science* 13 December 1991, 1643-1647.), a single chain fragment (scFv) of the monoclonal antibody G250, which with high selectivity has been shown to react with

20 a protein antigen on human renal carcinoma cells (Oosterwijk E, Ruiter DJ, Hoedemaeker PhJ, et al: Monoclonal antibody G250 recognizes a determinant present in renal-cell carcinoma and absent from normal kidney. *Int J Cancer* 38: 489-94, 1986.). G250 has been

25 extensively evaluated and has been applied in clinical trials (see the previous ref.).

30

Ad vectors can be made replication competent or incompetent for permissive cells. For tumor therapy,

35 replication competent Ad has the potential advantage that it can replicate and spread within the tumor (Miller R and Curiel DT: Towards the use of replicative adenoviral

vectors for cancer gene therapy, *Gene Therapy* 3: 557-559). This may theoretically result in an increase of the chosen effector mechanism over that obtainable with replication incompetent vectors. Furthermore, infectious virus may contribute to an anti tumor effect by cytopathogenic effects in infected cells as well as by evoking an anti viral immune response which may harm infected cells.

10 Construction, expression and evaluation of recombinant fibers

The aim has been to develop a universal method for the construction of functional Ad fibers with changed binding-specificity to make possible the construction of re-targeted Ad.

The adenovirus fiber peptide carries several biological functions which are necessary to retain in order to produce active virus particles. The following fiber features are deemed to be of key importance in the construction of functional recombinant fiber peptides:

- The ability to form parallel homotrimers. This function is carried by the N-terminal amino acid sequence of the wild type fiber knob and is necessary for the fiber to be able to bind to penton base and to form the functional cell binding knob.
- The ability to bind to penton base to form penton capsomeres. This function is carried by the wild type fiber tail.
- The ability to express a cell-binding ligand allowing for attachment to target cells. This function is carried by the wild type fiber knob.

- Since adenovirus is assembled in the nucleus of infected cells, the ability to be transported into the nucleus of infected cells is vital to virus formation. The nuclear localization signal is mainly, but perhaps not exclusively, carried by the wild type fiber tail.

In the first stage recombinant fibers are constructed and evaluated in vitro after cell-free expression in a coupled transcription/translation system. Analysis by SDS-PAGE and autoradiography is performed to reveal the presence of an open reading frame and give information on the size of the translated product. In the next stage recombinant fibers are cloned into Baculovirus and expressed in insect cells allowing for functional studies of the fibers. Such studies include ability to form trimers as evaluated by immunostaining with monoclonal antibody 2A6.36 which has been shown to react only with trimerised fibers (Shin Hong J and Engler JA: The amino terminus of the adenovirus fiber protein encodes the nuclear localization signal, *Virology* 185: 758-767, 1991), expression of functional ligand as evidenced by ability to bind to cells expressing the corresponding receptor and ability to bind to penton-base either in solution or on virions.

25

Recombinant fibers are constructed using methodology based on PCR (Clackson T, Güssow D and Jones PT: General application of PCR to gene cloning and manipulation, in PCR, A Practical Approach, Eds McPherson MJ, Quirke P and Taylor GR, IRL Press, Oxford, p 187, 1992), e.g. PCR-ligation-PCR (Alvaro Ali S, Steinkasserer A: PCR-ligation-PCR Mutagenesis: A Protocol for Creating Gene Fusions and Mutations, *BioTechniques* 18: 746-750, 1995)

30

and splicing by overlap extension (SOE) (Horton RM and Pease LR: Recombination and mutagenesis of DNA sequences using PCR, in McPherson MJ (ed), Directed Mutagenesis, IRL Press 1991, p 217.). Cloning is performed according to standard methods. Recombinant fibers are sequenced using Perkin Elmer ABI Prism and are expressed in mammalian cells and in insect cells and stained with monoclonal antibodies specific for fiber tail, trimeric fiber and the new cell-binding ligand. The following parameters are evaluated after immunostaining:

- trimerisation
- nuclear transportation
- expression of the new cell-binding ligand.

Finally, recombinant fibers are rescued into the Ad genome by a newly invented procedure described below and recombinant virus particles are produced.

The invention will be further illustrated with the following non-limiting examples:

Example 1:

Fiber peptides are made where the knob is replaced with an external trimerisation motif which is introduced after the TLWT motif which ends the shaft portion of the fiber. The purpose behind the introduction of an external trimerisation motif is two-fold: a) to remove the knob containing the native trimerisation signal but also the cell-binding part of the fiber, and b) simultaneously to supply the necessary trimerisation signal. Two different amino acid motifs have been used, i.e. the 36 amino acid "Neck Region Peptide" = NRP (SEQ ID NO: 1 in Sequence

10

listing) from human "Lung Surfactant Protein D" (. Hoppe
H-J, Barlow PN and Reid KBM: A parallel three stranded -
helical bundle at the nucleation site of collagen triple-
helix formation. *FEBS Letters* 344: 191-195 (1994).) and a
5 31 aa "Zipper" motif where the leucine residues on
positions 1 and 4 have been replaced with isoleucine
residues = pII (SEQ ID NO: 2 in Sequence listing)
(Harbury PB, Tao Zhang, Kim PS and Alber T: A Switch
Between Two-, Three-, and Four-Stranded Coiled Coils in
10 GCN4 Leucine Zipper Mutants. *Science* 262: 1401-1407,
1993.).

The DNA sequences for these trimerisation motifs are
synthesized, cloned and sequenced in the project.

15

To replace the cellbinding function of the knob a new
cellbinding ligand is introduced into the fiber in
addition to the external trimerisation amino acid motif.

20 To augment the efficiency of nuclear transportation of
recombinant fibers an external nuclear localisation
sequence is added to the fiber in some cases.

In further embodiments the fiber in addition contains
25 sequences which increase the survival of the fiber in the
cytosol of infected cells, thereby enhancing
transportation into the nucleus and virus assembly. Such
sequences are e.g. sequences that are present in the wild
type knob or in SEQ ID NO: 10 - 12.

30

The following types of fibers are constructed using the
methods mentioned above (see Fig 2). The sequence of the

11

wild type fiber is shown in the sequence listing as SEQ ID NO 14.

Type A

- 5 where the trimerisation motif is fused to the fiber gene downstream of the fiber shaft after the TLWT motif which constitutes the four first amino acids of the fiber knob or downstream of the second turn (Turn b) of any shaft repeat, the remaining shaft repeats having been removed.
- 10 The new cellbinding ligand is introduced downstream of the trimerisation signal with an amino acid linker motif being added between the trimerisation signal and the cellbinding ligand.

15 Type B

similar to type A but with a linker motif introduced immediately upstream of the trimerisation signal.

Type C

- 20 where the trimerisation motif is introduced after the first shaft repeat and in turn followed the shaft repeats 17 through 21. The new cellbinding ligand is introduced downstream of the trimerisation signal with an amino acid linker motif being added between the trimerisation signal
- 25 and the cellbinding ligand.

Type D

- where the cellbinding ligand is introduced between the restriction sites NheI and HpaI in the fiber shaft, with
- 30 an amino acid linker being added both upstream and downstream of the ligand.

Type D/ Δ

This is a variant of Type D where the fiber shaft downstream of the cellbinding ligand in Type D was removed. Type D and (D/ Δ) are constructed with the normal
5 knob and with the knob being replaced with an external trimerisation signal as in Types A and B.

Type E

which are similar to Type A but with part of the knob
10 being retained immediately upstream of the external trimerisation motif.

The following amino acid motifs are used as linkers in the above fiber constructs:

15

- SEQ ID NO: 3, derived from Pseudomonas exotoxin
- SEQ ID NO: 4, derived from tissue prothrombin activator
- SEQ ID NO: 5, derived from the hinge region of mouse immunoglobulin
- 20 • SEQ ID NO: 6, derived from Staphylococcal protein A
- SEQ ID NO: 7, derived from the hinge region of human IgG3
- SEQ ID NO: 8, derived from shaft repeat no 17 of human Ad5

25

Recombinant fibers are cloned into Baculovirus and expressed in Sf9 cells and/or cloned into the vector pSecTag and expressed in COS cells as secreted proteins. Expression is monitored by immunostaining with monoclonal
30 antibodies 4D2.5 (anti Ad5 fiber) and 2A6.36 (anti trimerised Ad5 fiber). Expression and trimerisation is

obvious in all recombinant fibers irrespective of length and trimerisation motif.

The various fibers which have been constructed and shown to be able to form trimers and express the new cell binding ligand are shown in Table 1. The results show that the invented technology is capable of generating trimerising fibers which express a new cellbinding ligand. It should therefore be possible to make functional virus with such fibers.

Table I. Results from immunostaining of different recombinant fibers

	Fiber	Detecting antibody				
		4D2	2A6	a-EGF	a-Ig	a-Id
15	Type A					
	A1 RGD	+	+			
	A1 EGF	+	+	+		
20	A1 G250 HK	+	+		+	+
	A1 G250 KH	+	+		+	+
	A1 G250 KHJCH2	+	+		+	+
	A1 V α LV β CB	+	+			
25	A7 RGD	+	+			
	A7 EGF	+	+	+		
	A7 G250 HK	+	+		+	+
	A7 G250 KH	+	+		+	+
	A7 G250 KHJCH2	+	+		+	+
30	A7 V α LV β CB	+	+			
	A7 IgG3 EGF	+	+	+		
	A7 (Gly4Ser)4 G250VKVH	+	+		+	+
35	A22 EGF	+	+	+		
	A22 RGD	+	+			
	Type B					
40	B (Gly4Ser)4 RGD	+	+			
	Type C					
	C IgG3 EGF	+	+	+		

14

C (Gly4Ser)4-
G250VKVH

+ + + +

Type D

5 N/D EGF + + +
N/D G250 HKCKy + + + +
F2/D EGF + + +
F3/D EGF + + +

10 Type D/Δ

F2 D/Δ G250 HKCK + + +
F2 D/Δ G250 HKCKy + + + +
F2 D/Δ EGF + + +
F3 D/Δ EGF + + +

15

Abbreviations used in Table 1:

2A6: antibody against trimerized fiber

4D2: antibody against fiber

a-EGF: antibody against epidermal growth factor

20 a-Id: anti idiotypic antibody specific for G250

a-Ig: antibody against mouse immunoglobulin

Cβ: Constant domain from β chain of T cell receptor
against MAGE1/HLA A1. SEQ ID NO: 11.

CH2: immunoglobulin heavy chain constant domain 2

25 EGF: epidermal growth factor

G250: monoclonal antibody specific for renal carcinoma

H: heavy chain variable sequence from G250 (SEQ ID NO:
15)

30 IgG3: amino acid linker derived from hinge region of
human IgG3, SEQ ID NO: 7

J: immunoglobulin joining chain sequence

K: light chain variable sequence from monoclonal antibody
G250 (SEQ ID NO: 16)

RGD: The amino acid sequence arginine-glycine-aspartic
acid

35

Vα: Variable domain from α chain of T cell receptor
against MAGE1/HLA A1. SEQ ID NO: 10

Vβ: Variable domain from β chain of T cell receptor
against MAGE1/HLA A1. SEQ ID NO: 12

Example 2:**Nuclear localization of recombinant fibers (Tables 2 and 3)**

5 Nuclear localization is assessed by immunostaining of fibers in Sf9 cells 24 hours after infection with the relevant Baculovirus clone. Some results are shown in Table 2 below. It is clear from these experiments that some recombinant fibers show a grossly impaired nuclear
10 localization in Sf9 cells despite the presence of the nuclear addressing signal in the fiber tail.

15	Table 2 Nuclear localization of native and selected recombinant fibers in Sf9 cells																
20	<table><tr><th data-bbox="267 1039 357 1066">Fiber</th><th data-bbox="454 1039 1235 1102">% of fiber-expressing Sf9 cells showing nuclear localization after infection</th></tr><tr><td data-bbox="267 1134 357 1161">Wild type</td><td data-bbox="885 1134 933 1161">100</td></tr><tr><td data-bbox="267 1171 357 1199">N/D EGF</td><td data-bbox="885 1171 933 1199">100</td></tr><tr><td data-bbox="267 1209 357 1236">A RGD</td><td data-bbox="812 1209 933 1236">App. 50</td></tr><tr><td data-bbox="267 1247 357 1274">A7 RGD</td><td data-bbox="795 1247 933 1274">App. 100</td></tr><tr><td data-bbox="181 1274 227 1302">25 A7 EGF</td><td data-bbox="795 1274 933 1302">App. 100</td></tr><tr><td data-bbox="267 1312 357 1339">A7 scTCR</td><td data-bbox="812 1312 933 1339">App. 50</td></tr><tr><td data-bbox="267 1371 357 1398">A7 G250 scFvs</td><td data-bbox="917 1371 933 1398">0</td></tr></table>	Fiber	% of fiber-expressing Sf9 cells showing nuclear localization after infection	Wild type	100	N/D EGF	100	A RGD	App. 50	A7 RGD	App. 100	25 A7 EGF	App. 100	A7 scTCR	App. 50	A7 G250 scFvs	0
Fiber	% of fiber-expressing Sf9 cells showing nuclear localization after infection																
Wild type	100																
N/D EGF	100																
A RGD	App. 50																
A7 RGD	App. 100																
25 A7 EGF	App. 100																
A7 scTCR	App. 50																
A7 G250 scFvs	0																

30 Recombinant and native fibers have also been expressed in COS cells, targeted for expression in the cytosol after cloning into the vector pcDNA 3.1. In this case it was expected that the fibers would be detected in the nucleus, due to the presence of the native nuclear
35 localization signal in the fiber tail. However, nuclear localization has so far only been detected in the wild type fiber and in fibers with single-chain T-cell

16

receptors, i.e. the fibers which have produced the most efficient virus (se below).

Since nuclear localization of fibers are crucial to virus assembly, an attempt is made to improve the efficiency of nuclear addressing by adding an external nuclear localization signal (NLS), in this case the SV40 large T-antigen NLS having the amino acid sequence SEQ ID NO: 9 (Fisher-Fantuzzi L and Vesco C: Cell-Dependent Efficiency of Reiterated Nuclear Signals in a Mutant Simian Virus 40 Oncoprotein Targeted to the Nucleus. *Mol Cell Biol*, 8:5495-5503, 1988). The external NLS sequence is added immediately up-stream of the RGD motif. It is found that the presence of the external NLS dramatically improved the nuclear localization in the cases where it has been investigated. In fact, as mentioned above the fiber constructs lacking the external NLS were undetectable in the transfected cells (Table 3).

20

Table 3

Nuclear localization of native and selected recombinant fibers in COS cells after targeting for expression in the cytosol

25

Fiber	Nuclear localization
Wild type	+
A V α LV β C β	+
A V α LV β C β Ck	+
A RGD	-
A NLS RGD	+
A7 RGD	-
A7 NLS RGD	+
A22 RGD	-

35

For abbreviations, se Table 1

The evidence given above support the hypothesis that recombinant fibers are poorly transported into the nucleus despite the presence of the intact tail region (see also below) and that this may possibly be corrected by the incorporation of an external NLS in the fiber construct.

Example 3:

10 METHOD FOR RESCUING OF RECOMBINANT FIBERS INTO VIRIONS

The wild type fiber in the Ad genome is exchanged for recombinant fibers by the following method (see Fig 3).

The plasmid pTG3602 (Chartier C, Degryse E, Gantzer M, Dieterlé A, Pavirani A and Mehtali M: Efficient generation of Recombinant Adenovirus Vectors by Homologous Recombination i Escherichia Coli, *J Virol*, 70: 4805-4810, 1996) containing the entire Ad5 genome as a PacI-PacI fragment is used as starting material. The approximate 9kb fragment of the genome between SpeI and PacI and containing the wild type fiber gene is cloned separately in pBluescript. From this fragment an approximate 3kb fragment between SacI and KpnI is further subcloned. A deletion of the native fiber gene with the exception of the N-terminal nucleotides upstream of the NdeI site of the fiber, between the NdeI site and the MunI site, which begins at base 38 after the stop codon of the fiber, is created in the 3kb fragment. The deleted sequence is replaced with SEQ ID NO: 13 which restores the NdeI and MunI sites and the wild type genome sequence between the fiber stop codon and the MunI site. In addition the added sequence, SEQ ID NO: 13, contains an XhoI site allowing for ligation of recombinant fibers

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into the fiber-deleted 3kb fragment (the 3 kb fiber shuttle) between NdeI and XhoI.

5 The 3 kb fiber shuttle with recombinant fiber is re-introduced into the 9 kb fragment cut with NheI using homologous recombination in E.coli (see ref. in previous passage). The resulting recombinant 9 kb fragment is finally excised from the vector with SpeI and PacI and joined to the isolated 27 kb fragment by Cosmid cloning.

10

The presence of an insert of the expected properties is verified in all cosmid clones by PCR. Cosmid clones are also restricted with Hind III and the presence of restriction fragments of the expected size verified on
15 gels.

Recombinant Ad genomes are isolated after restriction with Pac 1 and used to transfect suitable cells. The occurrence of plaques is determined by microscopic
20 inspection of the transfected cell cultures.

Supernatants are harvested from primarily transfected cultures and used to infect secondary cultures. The occurrence of cytopathogenic effects and plaques are
25 monitored by microscopy.

The particular fiber constructs that have been successfully rescued into virus are shown in figure 4a and 4b.

Conclusion:

For gene therapy to be useful for treatment of human
5 diseases there is a need for injectable vectors with
ability to target specific cells or a specific tissue
(Miller N and Vile R: Targeted vectors for gene therapy.
FASEB J, 9: 190-199, 1995).

10 The present invention describes methods whereby knobless,
trimerisation-competent fibers with new cellbinding
ligands can be created and rescued into virus and have
identified locations within the fiber-shaft which
tolerates inserts of foreign ligands. The importance of
15 intracellular trafficking of recombinant fibers has also
been identified. Recombinant virus made using the
invented technology should be highly useful in human
medicine. Virtually unlimited opportunities for targeted
gene-therapy may be developed by the combination of the
20 technology described here and the identification of cell-
binding ligands by phage-display.

So far trimerisation-competent fibers with a human scTCR
have been and rescued into functional virus. Since single
25 chain antibodies are large and highly complex peptides it
seems highly likely that also other scAbs and cell-
binding ligands, e.g. peptides identified from peptide
libraries by means of phage-display, could be
incorporated into Ad-fibers and rescued into virus using
30 the same technology.

There are many ways in which Ad, made re-targeted by the
present invention, may be applied to human gene therapy.

In the case of tumor diseases, the following options exist:

- I. Use of vectors to introduce transgenes into tumors,
- 5 such as
- anti onco genes
 - "suicide" genes
 - genes for immune modulatory substances or tumor antigens
- 10 • genes for anti angiogenetic factors
- II. Use of infectious virus. This has the added value over the use of non replicating vectors that virus can spread from cell to cell within a tumor, thereby
- 15 multiplying the initial hit on the tumor. Tumor cell destruction may occur not only by the cell-destroying mechanism engineered into the vector but also by the cell destruction which is associated with the virus infection per se and by the attack of the body's immune response on
- 20 the virus infected cells. This principle has already been tested in man through the direct intra-tumoral injection of an adenovirus which has been made gene manipulated to replicate only in p53 mutant tumor cells. The experience from these limited trials on large "head-and-neck" tumors
- 25 are partially encouraging with a complete regress of 2/11 treated tumors which are otherwise resistant to any form of known treatment.

Claims

1. Recombinant adenovirus with changed tropism, characterized in that the native pentone fibre, comprising a fibre tail, a fibre shaft and a fibre knob including a trimerisation motif, has been changed in that the native knob containing the cell binding structure and the native trimerisation motif has been removed and a new cellbinding ligand and an external trimerisation motif have been introduced into the virus fiber.
2. Adenovirus according to claim 1, chracterized in that said structural modification has been performed by DNA technology at the gene level or by chemical or immunological means at the virus level.
3. Adenovirus according to claim 1 which is either replication competent or replication in-competent.
4. Adenovirus according to claim 1, characterized in that the new cellbinding ligand has been introduced into the fiber shaft.
5. Adenovirus according to claim 1, characterized in that the new cell binding ligand has been introduced downstream of the fiber shaft repeats.
6. Adenovirus according to claim 4 characterized in that the new cellbinding ligand has been introduced between the restriction sites Nhe1 and Hpa1 in the fiber shaft.
7. Adenovirus according to claim 4, characterized in that amino acid linkers have been introduced upstream and downstream of the cellbinding ligand.

8. Adenovirus according to claim 4, characterized in that the shaft repeats downstream of the restriction site HpaI have been removed.
- 5 9. Adenovirus according to claim 1, characterized in that an amino acid linker motif has been added between the fiber shaft and the trimerisation motif and/or between the trimerisation motif and the cellbinding ligand as a linker.
- 10 10. Adenovirus according to claim 9, characterized in that the amino acid linker motif is any of the following: SEQ ID NO: 3, derived from Pseudomonas exotoxin; SEQ ID NO: 4, derived from tissue prothrombin activator; SEQ ID NO: 5, derived from the hinge region of mouse immunoglobulin; SEQ ID NO: 6, derived from Staphylococcal protein A; SEQ ID NO: 7, derived from the hinge region of human IgG3 ; SEQ ID NO: 8, derived from shaft repeat 17 of human Ad5.
- 20 11. Adenovirus according to any of the claims 1 - 10, characterized in that the new cellbinding ligand is any cellbinding peptide.
- 25 12. Adenovirus according to claim 11, characterized in that the cell binding ligand is a monoclonal antibody or a fragment thereof whether as a single chain fragment or Fab, a T cell receptor or a fragment thereof, an integrin binding peptide such as RGD or a growth factor such as
- 30 Epidermal Growth Factor.
13. Adenovirus according to claim 12, containing any of the sequences SEQ ID NO: 10 - 12.
- 35 14. Adenovirus according to claim 12, characterized in that the single chain fragment is a single chain fragment of the monoclonal antibody G250 with heavy chain variable

region with SEQ ID NO: 15 and light chain variable region with SEQ ID NO: 16.

15. Adenovirus according to claim 1 characterized in
5 that the external trimerisation motif is an α -helical coiled coil motif ,or any other peptide capable of rendering functionally trimerised fibers.

16. Adenovirus according to claim 15, characterized in
10 that the external trimerisation motif is the neck region peptide of human lung surfactant protein D, SEQ ID NO: 1 or a 31 aa "Zipper" motif where the leucine residues on positions 1 and 4 have been replaced with isoleucine residues, SEQ ID NO: 2.

15

17. Adenovirus according to any of the preceding claims characterized in that an external nuclear localisation signal (NLS) has been introduced in the fiber.

20 18. Adenovirus according to claim 17, characterized in that the NLS is the SV40 large-T antigen NLS.

19. Adenovirus according to any of the preceding claims characterized in that the fiber in addition contains
25 sequences which increase the survival of the fiber in the cytosol of infected cells, thereby enhancing transportation into the nucleus and virus assembly.

20. Adenovirus according to claim 19, characterized in
30 that the sequences are present in the wild type knob.

21. Adenovirus according to claim 20, characterized in that the sequences are present in SEQ ID NO: 10 - 12.

35 22. Adenovirus according to claims 1 - 21 for the treatment of human diseases, either in vivo or by in vitro methods.

23. A method of producing a recombinant adenovirus with changed tropism, comprising:

5

I. rescuing recombinant adenovirus fibres into the adenovirus genome by the following steps:

a) subcloning of a 9kb fragment (from SpeI to end of genome),

10 b) further subcloning of a 3kb fragment between SacI and KpnI,

c) deletion of the native fibergene coding for the native penton fibre between NdeI and MunI and replacing the missing sequence with the sequence SEQ ID NO: 13

15 containing an XhoI site;

d) ligation of recombinant fiber gene coding for between NdeI and XhoI of construct under c) above;

e) re-introduction of construct under d) above into the 9 kb fragment cut with NheI using homologous

20 recombination in E. coli;

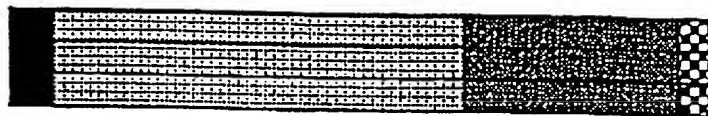
f) isolation of the recombinant 9 kb fragment under e) and re-creation of the adenovirus genome by joining 9 kb fragment to the 27 kb fragment from the beginning of the genome to the SpeI site by Cosmid cloning; and

25

II. transfecting a cell with the adenovirus obtained in step f) to enable said cell to express the recombinant adenovirus.

1/5

1 Wild type fiber trimer

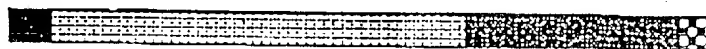
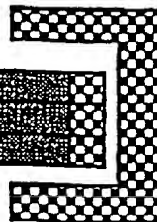


2 Fiber-penton interaction

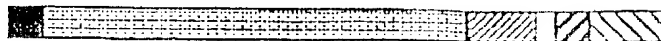


Mediates
Internalisation of
virus via Integrins
on the cell surface

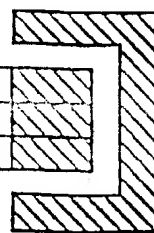
3 CAR Receptor-
fiber interaction
mediates binding
to the cell surface



4 Recombinant fiber



5 Cellular receptor
binding to new
ligand on
recombinant fibers



Code

- Fiber tail
- Fiber shaft
- Fiber knob
- New trimerisation motif

- Linker motif
- New cellbinding ligand
- Native trimerisation motif
- Nuclear localisation signal

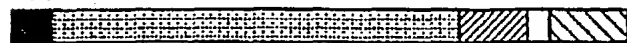
Fig. 1

2/5

Wild type fiber.



Type A



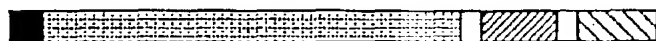
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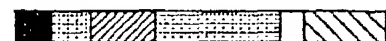
Type A7. Shaft repeats 1-7



Type B



Type C



R1 R18-21

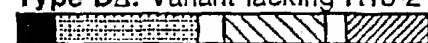
Type D



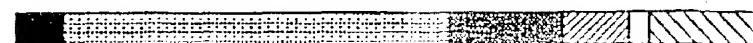
R 1-8

R18-21

Type DΔ. Variant lacking R18-21



Type E. Contains A, B and C sheets of knob.



Code



Fiber tail



Fiber shaft



Fiber knob



New trimerisation motif



Linker motif



New cellbinding ligand



Native trimerisation motif

R = Shaft repeat

Linker motifs

ASGGPE = Pseudo exo

ASEGNSD = TPA

ASTPEPDP = Ab Hinge, mouse

AKKLNDQAQPKSD from SpA

TPLGDTTHTSG = Upper hinge from human IgG3
(GGGGS)₄

Fig. 2

3/5

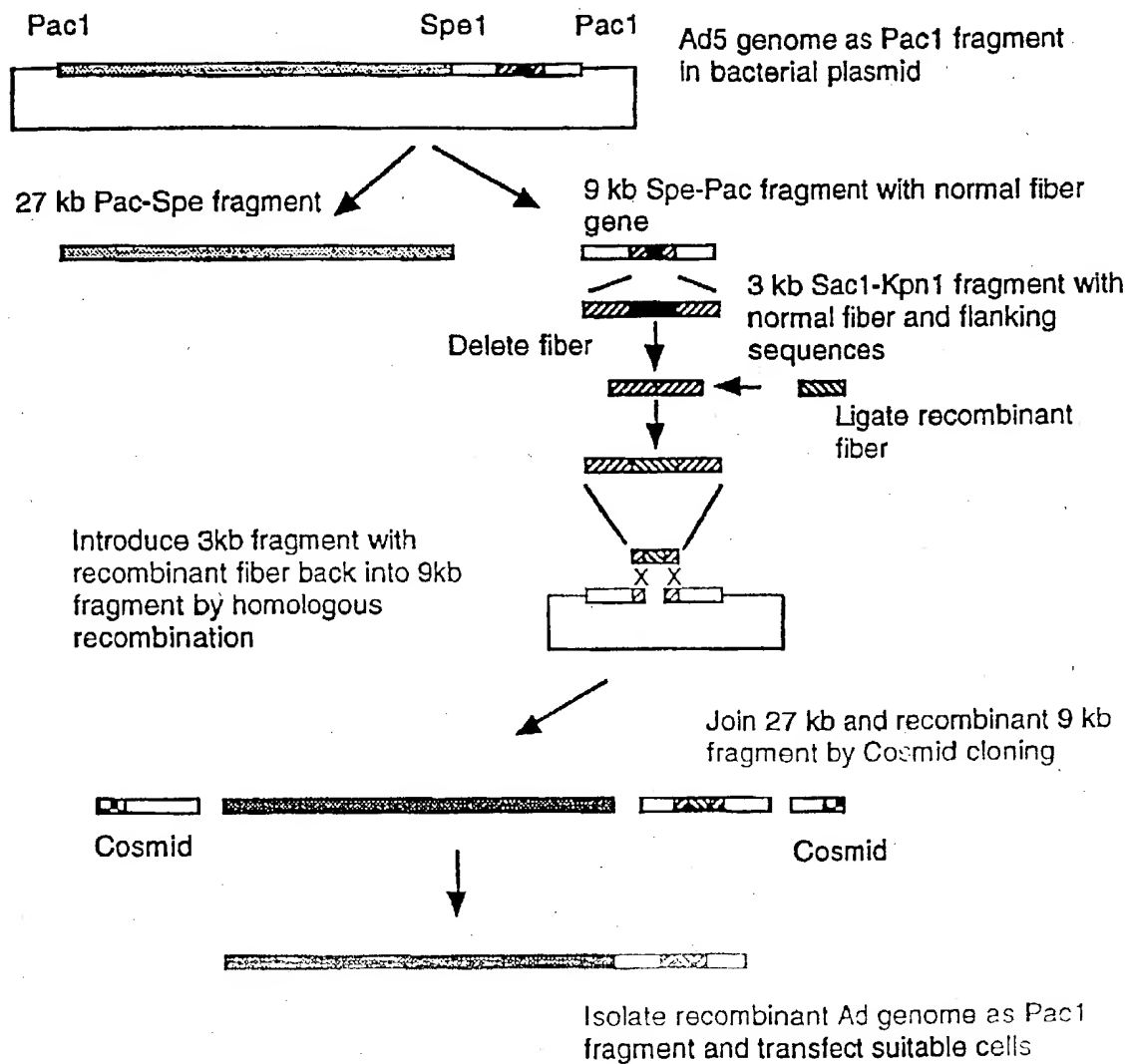


Fig. 3

4/5

Type A*Time in days for development of
plaques on primarily transfected
cells*

A1 Knob



14

A7 Knob



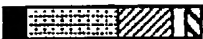
11-14

A1 RGD



23

A7 RGD



16

A7 NLS RGD



Not known

A1 EGF



Uncertain

A1 scFv



Uncertain

A1 scTCR



Uncertain

A7 scTCR



Uncertain



As WT



As WT



As WT

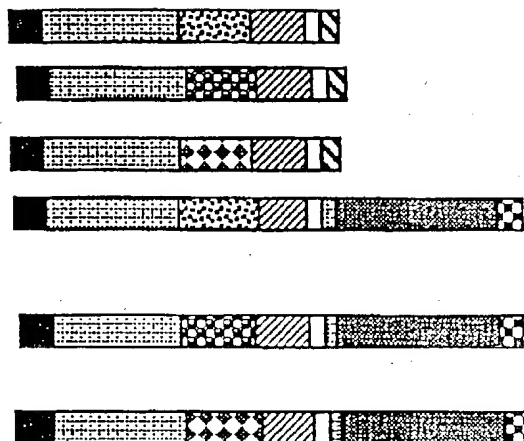
Color code

Fiber tail	Single chain antibody
Fiber shaft	V α
Fiber knob	V β
New trimerisation motif	C β
Linker motif	C κ
EGF	RGD
Native trimerisation motif	Nuclear localization signal

Fig. 4a

5/5

*Time in days for development of
plaques on primarily transfected
cells*

Type B

21

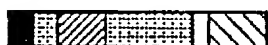
23

No plaques

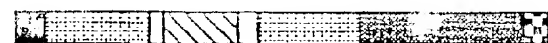
12

11

No plaques

Type C

Uncertain

Type D

15



Uncertain

Code

- | | |
|----------------------------|--|
| Fiber tail | (Gly4Ser)4 linker |
| Fiber shaft | Turn b from repeat 17 of Ad5 fiber shaft |
| Fiber knob | Turn b from repeat 22 of Ad5 fiber shaft |
| New trimerisation motif | |
| Linker motif | |
| EGF | |
| Native trimerisation motif | |

Fig. 4b

Sequence listing

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<160> 16

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<301> Hoppe HJ, Barlow PN, Reid KBM

<302> A parallel three stranded α -helical bundle at the nucleation site of collagen triple-helix formation

<303> FEBS Letters

<304> 344

<306> 191-195

<307> 1994

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Pro Asp Val Ala Ser Leu Arg Gln Gln Val Glu Asp Leu Gln Gly
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35

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 <301> Harbury PB, Zhang T, Kim PS, Albert T
 <302> A switch between two-, three-, and four-stranded coiled coils in GCN4 leucine zipper mutants
 <303> Science
 <304> 262
 <306> 1401-1407
 <307> 1993-11-26
 <400> 2

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 <301> Brinkmann U, Buchner J, Pastan I
 <302> Independent domain folding of Pseudomonas exotoxin and single chain immunotoxins: Influence of interdomain connections
 <303> Proc Natl Acad Sci US
 <304> 89

<306> 3075-3079

<307> 1992

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<302> Independent domain folding of Pseudomonas exotoxin and single chain immunotoxins: Influence of interdomain connections

<303> Proc Natl Acad Sci US

<304> 89

<306> 3075-3079

<307> 1992

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- <302> Independent domain folding of Pseudomonas exotoxin and single chain immunotoxins: Influence of interdomain connections
- <303> Proc Natl Acad Sci US
- <304> 89
- <306> 3075-3079
- <307> 1992
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1 5

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- <213> Homo sapiens

<301> Dangi JL, Wensel TG, Morrison SL, Streier L, Herzenberg LA and Oi
T

<302> Segmental flexibility and complement fixation of genetically
engineered chimeric human, rabbit and mouse antibodies

<303> EMBO Journal

<304> 7

<306> 1989

<307> 1988

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Thr Pro Leu Gly Asp Thr Thr His Thr Ser Gly
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<213> Adenovirus type 5

<301> Stouten PFW, Sander C, Ruigrok WH, Cusack S

<302> New triple-helical model for the shaft of the adenovirus fibre

<303> Journal of molecular biology

<304> 226

<306> 1073-1084

<307> 1992

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- <210> 9
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 <213> Simian virus 40
 <301> Fisher-Fantuzzi L and Vesco C 8:5495-5503, 1988
 <302> Cell-Dependent Efficiency of Reiterated Nuclear Signals in a Mutant
 Simian Virus 40 Oncoprotein Targeted to the Nucleus
 <303> Molecular Cell Biology
 <304> 8
 <306> 5495-5503
 <307> 1992
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 35 40 45

7

Arg Arg Asn Ser Phe Asp Glu Gln Asn Glu Ile Ser Gly Arg Tyr Ser
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 Ser Gln Val Val Asp Ser Ala Val Tyr Phe Cys Ala Leu Gly Gly Val
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 35 40 45
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 65 70 75
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 80 85 90
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 35 40 45
 Tyr Tyr Asn Gly Glu Glu Arg Ala Lys Gly Asn Ile Leu Glu Arg Phe
 50 55 60 65
 Ser Ala Gln Gln Phe Pro Asp Leu His Ser Glu Leu Asn Leu Ser Ser
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 Leu

<210> 13

<211> 52

<212> DNA

<213> Artificial sequence

<223> Sequence replacing the fiber gene sequence which was deleted
 between the NdeI restriction site in the fiber tail and the MunI site
 which begins at base 38 after the stop codon in the fiber. The sequence
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 between the fiber stop codon and the MunI site. In addition the added
 sequence contains an XhoI site allowing for the ligation of
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 <213> Human adenovirus type 5
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 <223> 1-129 Fiber tail
 130-1200 Fiber shaft
 1201-1746 Fiber knob
 <400> 14

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tat gac acg gaa acc ggt cct cca act gtg cct ttt ctt act cct ccc      96
Tyr Asp Thr Glu Thr Gly Pro Pro Thr Val Pro Phe Leu Thr Pro Pro
      20        25        30
ttt gta tcc ccc aat ggg ttt caa gag agt ccc cct ggg gta ctc tct      144
Phe Val Ser Pro Asn Gly Phe Gln Glu Ser Pro Pro Gly Val Leu Ser
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      65        70        75        80
caa aat gta acc act gtg agc cca cct ctc aaa aaa acc aag tca aac      288
Gln Asn Val Thr Thr Val Ser Pro Pro Leu Lys Lys Thr Lys Ser Asn
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      100       105       110
act gtg gct gcc gcc gca cct cta atg gtc gcg ggc aac aca ctc acc      384
Thr Val Ala Ala Ala Ala Pro Leu Met Val Ala Gly Asn Thr Leu Thr
      115       120       125
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      130       135       140
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aaa gag ccc att tat aca caa aat gga aaa cta gga cta aag tac ggg Lys Glu Pro Ile Tyr Thr Gln Asn Gly Lys Leu Gly Leu Lys Tyr Gly 195 200 205	624
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glt aac cta agc act gcc aag ggg ttg atg ttt gac gct aca gcc ata Val Asn Leu Ser Thr Ala Lys Gly Leu Met Phe Asp Ala Thr Ala Ile 325 330 335	1008
gcc att aat gca gga gat ggg ctt gaa ttt ggt tca cct aat gca cca Ala Ile Asn Ala Gly Asp Gly Leu Glu Phe Gly Ser Pro Asn Ala Pro 340 345 350	1056
aac aca aat ccc ctc aaa aca aaa att ggc cat ggc cta gaa ttt gat Asn Thr Asn Pro Leu Lys Thr Lys Ile Gly His Gly Leu Glu Phe Asp 355 360 365	1104
tca aac aag gct atg gtt cct aaa cta gga act ggc ctt agt ttt gac Ser Asn Lys Ala Met Val Pro Lys Leu Gly Thr Gly Leu Ser Phe Asp 370 375 380	1152
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11

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 435 440 445
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 Gly Val Leu Leu Asn Asn Ser Phe Leu Asp Pro Glu Tyr Trp Asn Phe
 465 470 475 480
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 Arg Asn Gly Asp Leu Thr Glu Gly Thr Ala Tyr Thr Asn Gly Val Gly
 485 490 495
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 Phe Met Pro Asn Leu Ser Ala Tyr Pro Lys Ser His Gly Lys Thr Ala
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 aaa agt aac att gtc agt caa gtt tac tta aac gga gac aaa act aaa 1584
 Lys Ser Asn Ile Val Ser Gln Val Tyr Leu Asn Gly Asp Lys Thr Lys
 515 520 525
 cct gta aca cta acc att aca cta aac ggt aca cag gaa aca gga gac 1632
 Pro Val Thr Leu Thr Ile Thr Leu Asn Gly Thr Gln Glu Thr Gly Asp
 530 535 540
 aca act cca agt gca tac tet atg tca ttt tca tgg gac tgg tet ggc 1680
 Thr Thr Pro Ser Ala Tyr Ser Met Ser Phe Ser Trp Asp Trp Ser Gly
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 cac aac tac att aat gaa ata ttt gcc aca tcc tet tac act ttt tca 1728
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<212> PRT

<213> Mus musculus

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12

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 Ala Ala Ile Asn Ser Asp Gly Gly Ile Thr Tyr Tyr Leu Asp Thr Val
 50 55 60
 Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Thr Leu Tyr
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 Leu Gln Met Ser Ser Leu Lys Ser Glu Asp Thr Ala Leu Phe Tyr Cys
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 Ala Arg His Arg Ser Gly Tyr Phe Ser Met Asp Tyr Trp Gly Gln Gly
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 Thr Ser Val Thr Val Ser Ser Gly Ser
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<212> PRT

<213> Mus musculus

<400> 16

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 Asp Arg Val Ser Ile Thr Cys Lys Ala Ser Gln Asn Val Val Ser Ala
 20 25 30
 Val Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ser Pro Lys Leu Leu Ile
 35 40 45
 Tyr Ser Ala Ser Asn Arg Tyr Thr Gly Val Pro Asp Arg Phe Thr Gly
 50 55 60
 Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Asn Met Gln Ser
 65 70 75 80
 Glu Asp Leu Ala Asp Phe Phe Cys Gln Gln Tyr Ser Asn Tyr Pro Trp
 85 90 95
 Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys Arg Ala Asp Ala Ala
 100 105 110
 Pro Thr Val Ser
 115

INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 00/01390

A. CLASSIFICATION OF SUBJECT MATTER

IPC7: C07K 14/075, C12N 7/00, C12N 15/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7: C07K, C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 9720051 A2 (GENVEC, INC.), 5 June 1997 (05.06.97), page 7, line 7 - line 17; page 11, line 22 - page 13, line 16; page 14; line 22 - page 17, line 22, page 33, line 31 - page 34, line 17; page 45, line 13 - line 27; page 77, line 1 - line 9; abstract	1-6,9-12,15, 19-20,22-23
X	WO 9626281 A1 (GENVEC, INC. ET AL), 29 August 1996 (29.08.96), page 9, line 4 - line 13; page 13, line 23 - line 35; page 16, line 1 - line 6, page 23, line 15 - line 19; example 6	1-4,11-12, 15,22
A		23

☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:	"I" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier application or patent but published on or after the international filing date	"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"I" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search	Date of mailing of the international search report
2 November 2000	13 -11- 2000
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Henrik Nilsson/ELY Telephone No. +46 8 782 25 00

INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 00/01390

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 9720575 A1 (THE UNIVERSITY OF ALABAMA AT BIRMINGHAM RESEARCH FOUNDATION), 12 June 1997 (12.06.97), figure 8, example 8 --	23
A	JOURNAL OF VIROLOGY, Volume 71, No 6, June 1997, Susan C. Stevenson et al, "Selective Targeting of Human Cells by a Chimeric Adenovirus Vector Containing a Modified Fiber Protein", page 4782 - page 4790, see page 4782, right-hand-column --	12
P,X	WO 9941359 A1 (THE UAB RESEARCH FOUNDATION), 19 August 1999 (19.08.99), page 4, line 12 - page 5, line 29; page 7, line 20 - line 23; page 8, line 26 - page 9, line 2, page 9, line 19 - line 20; claims 1-15 -- -----	1-4,11-12, 15-16,22

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE00/01390

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This international Searching Authority found multiple inventions in this international application, as follows:

See extra sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE00/01390

According to PCT Rules 13.1 and 13.2, an international application shall relate to one invention only or a group of inventions linked by one or more of the same corresponding "special technical features", i.e. features that define a contribution which each of the inventions makes over the prior art.

In Your application the following inventions have been found:

1. A recombinant adenovirus with modified tropism. The special technical feature of this invention is that the native knob containing the cell binding structure and the native trimerisation motif has been removed and a new cellbinding ligand and an external trimerisation motif has been introduced into the virus fiber. This invention is disclosed in claims 1-22 and has been searched.
2. A method in which the special technical feature is rescuing of recombinant adenovirus fibers into the adenovirus genome. This invention is disclosed in claim 23 and has been searched since the search could be performed within one search fee.

The two inventions are not linked by any common "special technical feature". Thus, the application lacks unity. Both inventions have been searched, since the search could be performed within one search fee.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

03/10/00

PCT/SE 00/01390

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